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INHIBITION OF ANGIOGENESIS BY HIGH MOLECULAR WEIGHT KININOGEN AND PEPTIDE ANALOGS THEREOF

Cross-Reference to Related Application

The benefit of U.S. provisional patent application 60/107,833
 5 filed November 10, 1998 is hereby claimed. The entire disclosure of
 application 60/107,833 is incorporated herein by reference.

Field of the Invention

The invention relates to therapeutic compounds and methods
 for inhibiting angiogenesis.

Background of the Invention

Angiogenesis

Angiogenesis is the process in which new blood vessels grow
 into an area which lacks a sufficient blood supply. Angiogenesis
 commences with the erosion of the basement membrane surrounding
 15 endothelial cells and pericytes forming capillary blood vessels. Erosion of
 the basement membrane is triggered by enzymes released by endothelial
 cells and leukocytes. The endothelial cells then migrate through the
 eroded basement membrane when induced by angiogenic stimulants. The
 migrating cells form a "sprout" off the parent blood vessel. The migrating
 20 endothelial cells proliferate, and the sprouts merge to form capillary loops,
 thus forming a new blood vessel.

Angiogenesis can occur under certain normal conditions in
 mammals such as in wound healing, in fetal and embryonic development,
 and in the formation of the corpus luteum, endometrium and placenta.
 25 Angiogenesis also occurs in certain disease states such as in tumor

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formation and expansion, or in the retina of patients with certain ocular disorders. Angiogenesis can also occur in a rheumatoid joint, hastening joint destruction by allowing an influx of leukocytes with subsequent release of inflammatory mediators.

5 The evidence for the role of angiogenesis in tumor growth was extensively reviewed by O'Reilly and Folkman in U.S. Pat. 5,639,725, the entire disclosure of which is incorporated herein by reference. It is now generally accepted that the growth of tumors is critically dependent upon this process. Primary or metastatic tumor foci are unable to achieve a size
10 of more than approximately 2 mm in the absence of neovascularization. Serial evaluation of transgenic mice predisposed to develop neoplasms has demonstrated that neovascularization of premalignant lesions precedes their evolution into tumors (Folkman *et al.*, *Nature* 339:58-61, 1989), and that inhibition of angiogenesis delays the growth of such lesions, as well as
15 their assumption of a malignant phenotype (Hanahan *et al.*, *Cell* 86:353-364, 1996). In humans, several studies have demonstrated that increased density of microvessels within a tumor is associated with a poor clinical outcome (Weidner *et al.*, *J Natl Cancer Inst* 84:1875-1887, 1992).

 An emerging paradigm is that proteolytic fragments of plasma
20 or extracellular matrix proteins regulate angiogenesis. To date, several polypeptides with such activities have been identified. These include angiostatin, which contains kringles 1-4 plasminogen (O'Reilly *et al.*, *Cell* 79:315-328, 1994), endostatin, a 20 kD C-terminal fragment of collagen XVIII (O'Reilly *et al.*, *Cell* 88:277-285, 1997), PEX, the hemopexin domain
25 of matrix metalloprotease 2 (Brooks *et al.*, *Cell* 92:391-400, 1998), the C-terminal 16 kD fragment of prolactin (Clapp *et al.*, *Endocrinol* 133:1292-1299, 1993) and a 29 kD fragment of fibronectin (Homandberg *et al.*, *Am J Pathol* 120:327-332; 1985). In addition, both intact thrombospondin 1 as well as peptides derived from its procollagen domain and properdin-like
30 type-1 repeats express potent anti-angiogenic activity (Good *et al.*, *Proc Nat Acad Sci USA* 87:6624-6628, 1990); Tolsma *et al.*, *J Cell Biol* 122:497-511, 1993. In preclinical models, several of these fragments inhibited tumor growth, and some induced tumor regression and dormancy (Boehm *et al.*, *Nature* 390:404-407, 1997).

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High Molecular Weight Kininogen

High molecular weight kininogen (HK) is a 120 kD glycoprotein containing heavy and light chains, comprised of domains 1 through 3, and 5 and 6, respectively (Kaplan *et al.*, *Blood* 70:1-15, 1987).
5 The heavy and light chains are linked by domain 4, which contains bradykinin, a nonapeptide which mediates several events including NO-dependent vasodilation (Weimer *et al.*, *J Pharm Exp Therapeutics* 262:729-733, 1992). HK (also referred to as "single chain high molecular weight kininogen") binds with high affinity to endothelial cells, where it is cleaved
10 to two-chain high molecular weight kininogen (HK_a) by plasma kallikrein. Bradykinin is released from HK through cleavage mediated by plasma kallikrein (Kaplan *et al.*, *Blood* 70:1-15, 1987). This event occurs on the surface of endothelial cells following the activation of prekallikrein to kallikrein by an endothelial cell protease (Motta *et al.*, *Blood* 91:515-528,
15 1998). Cleavage of HK to form HK_a and release bradykinin occurs between Lys(362) and Arg(363). HK_a contains a 62 kD heavy chain and a 56 kD light chain linked by a disulfide bond.

Conversion of HK to HK_a is accompanied by a dramatic structural rearrangement, which has been demonstrated using rotary
20 shadowing electron microscopy (Weisel *et al.* *J. Biol Chem* 269:10100-10106, 1994). HK_a, but not HK, has been shown to inhibit the adhesion of endothelial and other cell types to vitronectin (Asakura, *J. Cell Biol* 116:465-476, 1992).

Although the binding of HK to endothelial cells has been well
25 characterized, comparatively little attention has been devoted to endothelial cell binding of HK_a. Furthermore, although binding of bradykinin to endothelial cells induces well-defined responses, functional consequences of the direct binding of HK_a have not been reported.

Summary of the Invention

30 The compounds of the present invention are in the form of peptides which possess anti-angiogenic activity.

In all embodiments, the peptide may optionally comprise an amino-terminal and/or carboxy-terminal protecting group.

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A compound of the formula X_1 -His-Lys-X-Lys- X_2 (hereinafter "X₁-His-Lys-X-Lys- X_2 peptide") is provided wherein

X is any amino acid,

5 X₁ is from zero to twelve amino acids, more preferably from zero to six amino acids, most preferably from zero to three amino acids, and

X₂ is from zero to twelve amino acids, more preferably from zero to six amino acids, most preferably from zero to three amino acids.

10 Preferably, X is an amino acid having a nonpolar side chain, i.e., Ala, Leu, Ile, Val, Pro, Phe, Trp, or Met; or X is an amino acid having a polar side group which is uncharged at pH 6.0 to 7.0, the zone of physiological pH, i.e., Ser, Thr, Tyr, Asn, Gln, Cys, or Gly. Most preferably, X is Asn, Phe or His.

15 Preferred compounds comprise fragments of HK. In one group of such preferred compounds,

X₁ is

(i) zero amino acids, or

20 (ii) the segment His-Gly-His-Glu-Gln-Gln-His-Gly-Leu-Gly-His-Gly (SEQ ID NO:1) , or N-terminal truncation fragment thereof containing at least one amino acid, and

X₂ is

(i) zero amino acids, or

25 (ii) the segment Leu-Asp-Asp-Asp-Leu-Glu-His-Gln-Gly-Gly-His-Val (SEQ ID NO:2) , or C-terminal truncation fragment thereof containing at least one amino acid.

In another group of such preferred compounds,

30 X₁ is

(i) zero amino acids, or

(ii) the segment Gly-His-Lys-His-Lys-His-Gly-His-Gly-His-Gly-Lys (SEQ ID NO:3) or N-terminal

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truncation fragment thereof containing at least one amino acid, and

X₂ is

- 5 (i) zero amino acids, or
(ii) the segment Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys-Thr (SEQ ID NO:4) or C-terminal truncation fragment thereof containing at least one amino acid.

10 According to a further preferred embodiment of the invention, the compound has a substantial amino acid homology to either the amino acid sequence His-Gly-His-Glu-Gln-Gln-His-Gly-Leu-Gly-His-Gly-His-Lys-Phe-Lys-Leu-Asp-Asp-Asp-Leu-Glu-His-Gln-Gly-Gly-His-Val (SEQ ID NO:5), or the amino acid sequence Gly-His-Lys-His-Lys-His-Gly-His-Gly-His-Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys-Thr (SEQ ID NO:6).

Exemplary and preferred compounds include:

- (a) His-Gly-His-Glu-Gln-Gln-His-Gly-Leu-Gly-His-Gly-His-Lys-Phe-Lys-Leu-Asp-Asp-Asp-Leu-Glu-His-Gln-Gly-Gly-His-Val (SEQ ID NO:5);
- 20 (b) Gly-His-Lys-Phe-Lys-Leu-Asp-Asp-Asp-Leu-Glu-His-Gln-Gly-Gly-His (SEQ ID NO:7);
- (c) Gly-His-Lys-His-Lys-His-Gly-His-Gly-His-Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys-Thr (SEQ ID NO:6);
- 25 (d) Lys-His-Gly-His-Gly-His-Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn (SEQ ID NO:8); and
- (e) His-Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys-Thr (SEQ ID NO:9).

30 The invention also encompasses a method of inhibiting endothelial cell proliferation comprising contacting endothelial cells with HK, HK_a or a X₁-His-Lys-X-Lys-X₂ peptide.

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The invention also encompasses a method of inducing apoptosis of endothelial cells comprising contacting endothelial cells with HK, HK_a or a X₁-His-Lys-X-Lys-X₂ peptide.

5 The invention is also a composition comprising a pharmaceutically effective carrier and HK, HK_a or a X₁-His-Lys-X-Lys-X₂ peptide.

10 The invention is also a method of inhibiting angiogenesis in a mammal in need of such treatment comprising administering to said mammal a therapeutically effective amount of a composition comprising a pharmaceutically effective carrier and HK, HK_a or a X₁-His-Lys-X-Lys-X₂ peptide. The mammal treated is preferably a human being.

Other aspects and advantages of the present invention are described in the drawings and in the following detailed description of the preferred embodiments thereof.

15 Abbreviations and Short Forms

The following abbreviations and short forms are used in this specification.

"bFGF" is recombinant human basic fibroblast growth factor.

20 "HK" means the mature form of high molecular weight kininogen, and any allelic variations thereof. By "mature" is meant the post-translationally-modified form of HK which results from cleavage of an eighteen amino acid leader from the initially translated molecule. All numbering with respect to amino acid positions of HK is from the N-terminus of the mature form as position 1. "HK" is synonymous with
25 "single chain HK", the mature form of high molecular weight kininogen prior to cleavage by kallikrein and the formation of two-chain high molecular weight kininogen.

30 "HK_a" means two-chain high molecular weight kininogen, the product of kallikrein cleavage of mature high molecular weight kininogen, and any allelic variations thereof.

"HDMVEC" means human dermal microvascular endothelial cells.

"HGF" means hepatocyte growth factor.

"HUVEC" means human umbilical vein endothelial cell

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"PDGF" is platelet-derived growth factor.

"TGF- β " is transforming growth factor- β .

"VEGF" means vascular endothelial cell growth factor.

"X₁-His-Lys-X-Lys-X₂ peptide" means a compound of the indicated formula wherein X, X₁ and X₂ are defined as above.

Amino Acid Abbreviations

The nomenclature used to describe polypeptide compounds of the present invention follows the conventional practice wherein the amino group is presented to the left and the carboxy group to the right of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxy-terminal groups, although not specifically shown, will be understood to be in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by a one-letter or three-letter designation, corresponding to the trivial name of the amino acid, in accordance with the following schedule:

	A	Alanine	Ala
	C	Cysteine	Cys
	D	Aspartic Acid	Asp
20	E	Glutamic Acid	Glu
	F	Phenylalanine	Phe
	G	Glycine	Gly
	H	Histidine	His
	I	Isoleucine	Ile
25	K	Lysine	Lys
	L	Leucine	Leu
	M	Methionine	Met
	N	Asparagine	Asn
	P	Proline	Pro
30	Q	Glutamine	Gln
	R	Arginine	Arg
	S	Serine	Ser
	T	Threonine	Thr
	V	Valine	Val
35	W	Tryptophan	Trp
	Y	Tyrosine	Tyr

Definitions

The following definitions, of terms used throughout the specification, are intended as an aid to understanding the scope and practice of the present invention.

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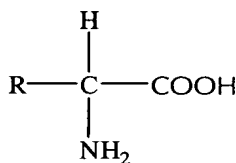
"**Angiogenesis**" means the generation of new blood vessels into a tissue or organ.

"**Apoptosis**" means a process of programmed cell death.

A "**peptide**" is a compound comprised of amino acid residues
5 covalently linked by peptide bonds.

The expression "**amino acid**" as used herein is meant to include both natural and synthetic amino acids, and both D and L amino acids. "**Natural amino acid**" means any of the twenty primary, naturally occurring amino acids which typically form peptides, polypeptides, and
10 proteins. "**Synthetic amino acid**" means any other amino acid, regardless of whether it is prepared synthetically or derived from a natural source. As used herein, "synthetic amino acid" also encompasses chemically modified amino acids, including but not limited to salts, derivatives (such as amides), and substitutions. Amino acids contained within the peptides of the present
15 invention, and particularly at the carboxy- or amino-terminus, can be modified by methylation, amidation, acetylation or substitution with other chemical groups which can change the peptide's circulating half life without adversely affecting their activity. Additionally, a disulfide linkage may be present or absent in the peptides of the invention, as long as anti-
20 angiogenic activity is maintained.

Amino acids have the following general structure:



Amino acids are classified into seven groups on the basis of the side chain
25 R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group. Peptides comprising a large
30 number of amino acids are sometimes called "**polypeptides**". The amino acids of the peptides described herein and in the appended claims are

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understood to be either D or L amino acids with L amino acids being preferred.

5 **"Homology"** means similarity of sequence reflecting a common evolutionary origin. Peptides or proteins are said to have homology, or similarity, if a substantial number of their amino acids are either (1) identical, or (2) have a chemically similar R side chain. Nucleic acids are said to have homology if a substantial number of their nucleotides are identical.

10 As used herein, **"protected"** with respect to a **terminal amino group** refers to a terminal amino group of a peptide, which terminal amino group is coupled with any of various amino-terminal protecting groups traditionally employed in peptide synthesis. Such protecting groups include, for example, acyl protecting groups such as formyl, acetyl, benzoyl, trifluoroacetyl, succinyl, and methoxysuccinyl; aromatic urethane protecting
15 groups such as benzyloxycarbonyl; and aliphatic urethane protecting groups, for example, tert-butoxycarbonyl or adamantyloxycarbonyl. See Gross and Mienhofer, eds., *The Peptides*, vol. 3, pp. 3-88 (Academic Press, New York, 1981) for suitable protecting groups.

20 As used herein, **"protected"** with respect to a **terminal carboxyl group** refers to a terminal carboxyl group of a peptide, which terminal carboxyl group is coupled with any of various carboxyl-terminal protecting groups. Such protecting groups include, for example, tert-butyl, benzyl or other acceptable groups linked to the terminal carboxyl group through an ester or ether bond.

25 **"Substantial amino acid sequence homology"** means an amino acid sequence homology greater than about 30 %, preferably greater than about 60%, more preferably greater than about 80%, and most preferably greater than about 90 %.

30  By **"N-terminal truncation fragment"** with respect to an amino acid sequence is meant a fragment obtained from a parent sequence by removing one or more amino acids from the N-terminus thereof.

By **"C-terminal truncation fragment"** with respect to an amino acid sequence is meant a fragment obtained from a parent

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sequence by removing one or more amino acids from the C-terminus thereof.

Description of the Figures

Fig. 1 shows the inhibition of endothelial cell proliferation over time following contact with HK_a.

~~Fig. 2 shows the concentration-dependent inhibition of endothelial cell proliferation by HK_a (black bars), HK (white bars) and low molecular weight kininogen (stippled bars). Low molecular weight kininogen is non-inhibitory.~~

Fig. 3 shows the ability of 30 nM HK_a to inhibit endothelial cell proliferation stimulated by a variety of growth factors.

Fig. 4 shows that HK_a inhibits proliferation of two types of endothelial cells (HUVEC and HDMVEC), but not human aortic smooth muscle cells (HASMC).

~~Fig. 5 shows the inhibition of endothelial cell proliferation as a function of HK_a concentration and cell density in the culture. White bars = 1,500 cells/well; diagonally hatched bars = 3,000 cells/well; grey bars = 6,000 cells/well; black bars = 12,000 cells/well; vertically hatched bars = 24,000 cells/well.~~

Fig. 6A shows endothelial cells after staining with 4',6'-diamidino-2-phenylindole hydrochloride. Fig. 6B shows endothelial cells upon staining with 4',6'-diamidino-2-phenylindole hydrochloride after four hours of exposure to HK_a.

Figs. 7A and 7B show DNA fragmentation in endothelial cells exposed to HK_a as a function of time (at 30 nM concentration) and concentration (at 12 hours), respectively.

Figs. 8A and 8B show athymic Ncr nude mice injected subcutaneously with chilled Matrigel[®] containing bFGF which resulted in formation of a visible "plug". The plug was photographed four days post implantation. Figs. 8C and 8D are similar to 8A and 8B except that the plug contained HK_a. The arrows in the figures point to the plug periphery. Plug vascularization is visible in Figs. 8A and 8B, but absent in Figs. 8C and 8D.

Detailed Description of the Invention

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The present invention is based upon the discovery that HK_a and peptide analogs of certain sites in the HK domain 5 inhibit endothelial cell proliferation and/or induce endothelial cell apoptosis. These activities confer upon HK_a and the X₁-His-Lys-X-Lys-X₂ peptides the ability to inhibit cytokine-driven angiogenesis *in vivo*.

Antiproliferative effects are observed at concentrations below 1.0 nM. The use among different laboratories of endothelial cells of different origin, and/or varying concentrations of endothelial cell growth factors, makes a direct comparison of the relative potency of HK_a and previously-reported anti-angiogenic polypeptides difficult. However, the observations made herein suggest that the *in vitro* potency of HK_a in this regard is similar to that of angiostatin (O'Reilly *et al.*, *Cell* 79:315-328, 1994), endostatin (O'Reilly *et al.*, *Cell* 88:277-285, 1997) and TSP-1 (Good *et al.*, *Proc Nat Acad Sci USA* 87:6624-6628, 1990). Furthermore, when the plasma concentration (670 nM) of the parent molecule, HK, is considered, it is apparent that the anti-angiogenic activity of HK_a and the X₁-His-Lys-X-Lys-X₂ peptides is physiologically significant.

The effects of HK_a, and thus the effects of the X₁-His-Lys-X-Lys-X₂ peptides also, are cell specific. No inhibition of the proliferation of either human aortic smooth muscle cells or HEK 293 cells by HK_a is observed. According to the assays utilized herein, HK_a potentially inhibits the proliferation of human umbilical vein and microvascular endothelial cells *in vitro* in response to various pro-angiogenic growth factors: bFGF, VEGF, hepatocyte growth factor, TGF-β and PDGF. Inhibition of endothelial cell proliferation is detected within 6 hours of exposure of the cells to HK_a, and is accompanied by morphologic and biochemical evidence of cell apoptosis.

Furthermore, as shown herein, HK_a is effective in an *in vivo* model of angiogenesis. HK_a inhibits the ingrowth of new blood vessels into a reconstituted extracellular matrix (Matrigel) containing the pro-angiogenic growth factor bFGF implanted subcutaneously into mice.

Without wishing to be bound by any theory, the observation that HK_a, but not single chain HK, inhibits endothelial cell proliferation suggests that the structural change which the molecule undergoes

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following kallikrein-mediated cleavage is important for expression of its anti-angiogenic activity.

The mature human HK amino acid sequence is set forth in the recent review by Colman and Schmaier, *Blood*, 90:3819-3843 (1997), for example. HK_a generated by plasma kallikrein cleavage of HK differs from HK in that it lacks the nine amino acid segment comprising HK amino acids 363-371. This segment is released from HK as the nonapeptide bradykinin. The two chains of HK resulting from the elimination of bradykinin remain linked by a disulfide bond between cysteine residues at positions 10 and 656 of mature HK. The N-terminal and C-terminal chains of HK_a generated by plasma kallikrein cleavage of HK and release of bradykinin are known as HK "heavy" and "light" chains, respectively. HK_a may be generated by treating HK with plasma kallikrein, according to well-known methods. HK_a is also commercially available. Furthermore, other enzymes, such as plasmin, chymotrypsin or matrix metalloproteases, for example, may degrade HK to release peptides similar to those described herein.

HK domain 5 spans HK residues 384-502. Located within domain 5 are two separate segments characterized by the sequence His-Lys-X-Lys. The first such segment occurs at position 457-460. The second segment occurs at position 488-491. HK_a-derived peptides containing the His-Lys-X-Lys sequence inhibit endothelial cell proliferation and are useful as anti-angiogenic agents.

The X₁-His-Lys-X-Lys-X₂ peptides of the present invention may be recombinant peptides, natural peptides, or synthetic peptides. They may also be chemically synthesized, using, for example, solid phase synthesis methods.

In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, e.g., the carbobenzyloxy group or the t-butyloxycarbonyl group, various coupling reagents (e.g., dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxy-succinimide, and the various cleavage reagents, e.g., trifluoroacetic acid

(TFA), HCl in dioxane, boron tris-(trifluoroacetate) and cyanogen bromide, and reaction in solution with isolation and purification of intermediates is well-known classical peptide methodology. The preferred peptide synthesis method follows conventional Merrifield solid-phase procedures. See
5 Merrifield, *J. Amer. Chem. Soc.* 85:2149-54 (1963) and *Science* 50:178-85 (1965). Additional information about the solid phase synthesis procedure can be had by reference to the treatise by Steward and Young (*Solid Phase Peptide Synthesis*, W.H. Freeman & Co., San Francisco, 1969, and the review chapter by Merrifield in *Advances in Enzymology* 32:221-296,
10 F.F. Nold, Ed., Interscience Publishers, New York, 1969; and Erickson and Merrifield, *The Proteins* 2:255 et seq. (ed. Neurath and Hill), Academic Press, New York, 1976. The synthesis of peptides by solution methods is described in Neurath et al., eds. (*The Proteins*, Vol. II, 3d Ed., Academic Press, NY (1976)).

15 Crude peptides may be purified using preparative high performance liquid chromatography. The amino terminus may be blocked according, for example, to the methods described by Yang *et al.* (*FEBS Lett.* 272:61-64 (1990)).

20 Peptide synthesis includes both manual and automated techniques employing commercially available peptide synthesizers. The X₁-His-Lys-X-Lys-X₂ peptides may be prepared by chemical synthesis and biological activity can be tested using the methods disclosed herein.

 Alternatively, the X₁-His-Lys-X-Lys-X₂ peptides may be prepared utilizing recombinant DNA technology, which comprises
25 combining a nucleic acid encoding the peptide thereof in a suitable vector, inserting the resulting vector into a suitable host cell, recovering the peptide produced by the resulting host cell, and purifying the polypeptide recovered. The techniques of recombinant DNA technology are known to those of ordinary skill in the art. General methods for the cloning and
30 expression of recombinant molecules are described in Maniatis (*Molecular Cloning*, Cold Spring Harbor Laboratories, 1982), and in Sambrook (*Molecular Cloning*, Cold Spring Harbor Laboratories, Second Ed., 1989), and in Ausubel (*Current Protocols in Molecular Biology*, Wiley and Sons, 1987), which are incorporated by reference. The complete cDNA of human
35 HK is reported, for example, by Takagi *et al.*, *J. Biol. Chem.* 260:8601-8609

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(1985), the entire disclosure of which is incorporated herein by reference. From this nucleic acid sequence, synthetic genes encoding HK_a-derived peptides may be synthesized directly on a DNA synthesizer, or may be synthesized as complementary oligonucleotides which are ligated together to form the synthetic gene.

The nucleic acids encoding HK_a-derived peptides may be operatively linked to one or more regulatory regions. Regulatory regions include promoters, polyadenylation signals, translation initiation signals (Kozak regions), termination codons, peptide cleavage sites, and enhancers. The regulatory sequences used must be functional within the cells of the vertebrate to be immunized. Selection of the appropriate regulatory region or regions is a routine matter, within the level of ordinary skill in the art.

Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoters may be prokaryotic or eukaryotic depending on the host. Among the prokaryotic (including bacteriophage) promoters useful for practice of this invention are lacI, lacZ, T3, T7, lambda Pr' Pl' and trp promoters. Among the eukaryotic (including viral) promoters useful for practice of this invention are ubiquitous promoters (e.g. HPRT, vimentin, actin, tubulin), intermediate filament promoters (e.g. desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (e.g. MDR type, CFTR, factor VIII), tissue-specific promoters (e.g. actin promoter in smooth muscle cells), promoters which respond to a stimulus (e.g. steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313, US 5,168,062 and 5,385,839, the entire disclosures of which are incorporated herein by reference.

Examples of polyadenylation signals that can be used in the present invention include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals.

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The X_1 -His-Lys-X-Lys- X_2 peptides prepared by either chemical synthesis or recombinant DNA technology may then be assayed for biological activity according to the assay methods described herein.

5 In some embodiments, the peptides of the present invention may be used in the form of a pharmaceutically acceptable salt.

Suitable acids which are capable of forming salts with the peptides include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acid and the like; and organic acids such as formic acid, acetic acid, 10 propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid and the like.

Suitable bases capable of forming salts with the peptides include inorganic bases such as sodium hydroxide, ammonium hydroxide, 15 potassium hydroxide and the like; and organic bases such as mono-, di- and tri-alkyl and aryl amines (e.g., triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (e.g., ethanolamine, diethanolamine and the like).

The present invention provides methods for inhibiting 20 angiogenesis. A preferred embodiment is a method of inhibiting the proliferation of endothelial cells, or obtaining apoptosis of such cells. Accordingly, HK, HK_a and/or one or more X_1 -His-Lys-X-Lys- X_2 peptides is administered to a patient in need of such treatment. A therapeutically effective amount of the drug may be administered as a composition in 25 combination with a pharmaceutically carrier. Although HK is considerably less anti-angiogenic than HK_a , it is possible that upon administration HK will be converted to HK_a , and may therefore serve as a prodrug for HK_a . In particular, it is believed that HK may be rapidly converted to HK_a in tumors, or further cleaved by tumor-derived enzymes to release peptides the same 30 or similar to the peptides disclosed herein.

The ability of HK_a to inhibit the proliferation of endothelial cells cultured on different extracellular matrix (ECM) proteins was determined. HK_a (10nM) potently inhibited the proliferation of HUVEC cultured on gelatin, laminin and Matrigel, though slightly less potent inhibition, largely 35 overcome by high concentrations of HK_a (50 nM), occurred when cell were

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cultured on fibronectin or vitronectin. Intermediate effects were observed when cells were cultured on fibrinogen, though cells cultured on collagen types I or IV were resistant to the antiproliferative activity of HK_a. In keeping with the results of proliferation assays, HK_a caused apoptosis of
5 endothelial cells cultured on gelatin, but not on collagen, and of cells cultured at low density, but not under confluent or near-confluent conditions. Without wishing to be bound by any theory, it appears that mature endothelial cells residing on an intact, collagen-rich basement membrane may be protected from HK_a-induced apoptosis, and that HK_a
10 might selectively target angiogenic endothelial cells in a protease-rich tumor milieu in which ECM is partially degraded.

Pharmaceutically acceptable carriers include physiologically tolerable or acceptable diluents, excipients, solvents, adjuvants, or vehicles, for parenteral injection, for intranasal or sublingual delivery, for
15 oral administration, for rectal or topical administration or the like. The compositions are preferably sterile and nonpyrogenic. Examples of suitable carriers include but are not limited to water, saline, dextrose, mannitol, lactose, or other sugars, lecithin, albumin, sodium glutamate cysteine hydrochloride, ethanol, polyols (propyleneglycol, ethylene,
20 polyethyleneglycol, glycerol, and the like), vegetable oils (such as olive oil), injectable organic esters such as ethyl oleate, ethoxylated isosteraryl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum methahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

25 The pharmaceutical compositions may also contain minor amounts of nontoxic auxiliary substances such as wetting agents, emulsifying agents, pH buffering agents, antibacterial and antifungal agents (such as parabens, chlorobutanol, phenol, sorbic acid, and the like). If desired, absorption enhancing or delaying agents (such as liposomes,
30 aluminum monostearate, or gelatin) may be used. The compositions can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions.

The compositions may be administered by any convenient
35 route which will result in delivery to the site of undesired angiogenesis in an

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amount effective for inhibiting that angiogenesis from proceeding. Modes of administration include, for example, orally, rectally, parenterally (intravenously, intramuscularly, intraarterially, or subcutaneously), intracisternally, intravaginally, intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray or aerosol. The compositions can also be delivered through a catheter for local delivery at a target site, or via a biodegradable polymer. The compositions may also be complexed to ligands, or antibodies, for targeted delivery of the compositions.

The compositions are most effectively administered parenterally, preferably intravenously or subcutaneously. For intravenous administration, they may be dissolved in any appropriate intravenous delivery vehicle containing physiologically compatible substances, such as sodium chloride, glycine, and the like, having a buffered pH compatible with physiologic conditions. Such intravenous delivery vehicles are known to those skilled in the art. In a preferred embodiment, the vehicle is a sterile saline solution. If the peptides are sufficiently small (e.g., less than about 8-10 amino acids) other preferred routes of administration are intranasal, sublingual, and the like. Intravenous or subcutaneous administration may comprise, for example, injection or infusion.

The compositions according to the invention can be administered in any circumstance in which inhibition of angiogenesis is desired. Disease states which may be treated include but are not limited to cancer, rheumatoid arthritis, and certain ocular disorders characterized by undesired vascularization of the retina. Because the peptides of the invention are anti-angiogenic, cancers characterized by the growth of solid tumors through angiogenesis of the tissue surrounding the tumor site may be treated according to the invention.

The amount of active agent administered depends upon the degree of the anti-angiogenic effect desired. Those skilled in the art will derive appropriate dosages and schedules of administration to suit the specific circumstances and needs of the patient. Typically, dosages are from about 0.1 to about 100, preferably from about 0.5 to about 50, most preferably from about 1 to about 20, mg/kg of body weight. The active agent may be administered by injection daily, over a course of therapy lasting two to three weeks, for example. Alternatively, the agent may be

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administered by continuous infusion, such as via an implanted subcutaneous pump.

Peptides which inhibit endothelial cell proliferation by at least 30%, more preferably by at least 50%, most preferably by at least 70%,
5 when incubated with such cells at a concentration of 50 μ M are preferred. For purposes of this preference, percent inhibition of proliferation is determined according to the procedure and formula set forth in Example 1, part A, below.

Examples

10 The present invention is illustrated by the following non-limiting examples.

Materials

The materials utilized in the Examples were sourced as follows. Tissue culture medium and reagents were obtained from
15 Mediatech (Herndon, VA). Fetal bovine serum was from Hyclone (Logan, Utah). Endothelial growth supplement was purified from bovine hypothalamii, as previously described (Maciag *et al.*, *Proc natl Acad Sci* 76:5674-5678, 1979). Recombinant human basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF) and hepatocyte
20 growth factor (HGF) were obtained from Collaborative Biomedical Products/Becton Dickinson (Bedford, MA). Platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β) were from R&D Systems (Minneapolis, MN). Gelatin was purchased from Sigma (St. Louis, MO). Single and two-chain high molecular weight kininogen were obtained from
25 Enzyme Research Labs (South Bend, IN). HK_a was >98% two-chain, as determined using 10% SDS-PAGE after reduction. Low molecular weight kininogen was purchased from American Research Products, (Belmont, MA). Bradykinin was from Peninsula Laboratories (Belmont, CA), and rabbit anti-bradykinin antiserum from Sigma. All HK_a preparations used in
30 these studies contained less than <0.01 EU/ml of endotoxin, as determined using the E-Toxate (Limulus Amoebocyte) assay (Sigma).

Synthetic Peptides

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Synthetic peptides were synthesized on a Rainin Symphony multiple peptide synthesizer, using Fmoc chemistry. All resins (AnaSpec) used for solid phase synthesis were Wang resins preloaded with the first amino acid. Fmoc amino acids were purchased from Perseptive Biosystems, with side chain protective groups as follows: trityl (Asn, Cys, Gln, and His), Boc (Lys and Trp), Ombu (Asp and Glu), T.U. (Ser, Thr and Tyr) and P.G. (Arg). Deprotection of the Fmoc group was performed in piperidine in dimethylformamide (DMF). Coupling was carried out done in HBTU in N-methylmorpholine/DMF as the activator. Standard synthesis cycles were 3 x 30" washes with DMF, 3 x 15" deprotection with piperidine, 6 x 20" DMF washes, 45 minute coupling with amino acid and activator followed by 3 x 30" DMF washes.

Peptides were cleaved off the solid phase support with cleavage cocktail consisting of 88:5:5:2 (TFA:water:phenol:triisopropylsilane). Cleavage was done on the synthesizer. Peptides were precipitated with ether, pelleted by centrifugation, washed three times with ether and then allowed to dry. HPLC was carried out on a Beckman HPLC system using Rainin Dynamax Reversed Phase columns and an acetonitrile gradient in water. The desired peptide was detected during elution by off line MALDI-TOF mass spectrophotometry using a Perseptive Biosystems Voyager instrument. Purified peptides were lyophilized and then reanalyzed by MALDI-TOF mass spectrophotometry.

Cell Culture Methods

The basic cell culture methods of the Examples are described as follows. Human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cells were isolated and cultured as previously described (Graham *et al.*, *Blood* 91:3300-7 1998). Human dermal microvascular endothelial cells (HDMVEC) were obtained from Clonetics (San Diego, CA) and cultured under identical conditions. All cells in these studies were of passage 3 or lower.

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Example 1**Effect of Single-Chain and Two-Chain High Molecular Weight Kininogen on Endothelial Cell Proliferation****A. Experimental**

5 To assess the effect of HK_a on endothelial cell proliferation, cells were suspended at a concentration of 30,000 cells/ml in Medium 199 (M199) containing 2% FCS. One hundred microliters of this suspension was then plated in individual wells of a 96 well microplate precoated with 1% gelatin. After incubation for 2 hours, at 37°C, to allow cells to adhere and spread, medium was removed and replaced with fresh M199 containing (i) 2% FCS, (ii) 10 µM ZnCl₂, (iii) 10 ng/ml bFGF, VEGF, HGF, TGF-β or PDGF and (iv) 50 µM HK or HK_a. Cells were then incubated for 10 48 hours at 37°C, at which time the relative numbers of cells in each well were determined using the Cell Titer[®] Aqueous cell proliferation assay (Promega, Madison, WI). Briefly, 20 µl of a 19:1 (V/V) mixture of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) was added to each well, and after an additional hour of incubation, A₄₉₀ was measured using a BioRad model EL311 microplate reader. The percent inhibition of cell 15 proliferation by HK_a was determined using the formula:

20 ~~$$\% \text{ inhibition} = (1 - [(A_{490 (+GF, HK_a)} - A_{490 (-GF)}) / (A_{490 (-GF)} - A_{490 (-GF)})]) \times 100$$~~

where (+GF) and (-GF) represent proliferation in the presence or absence of added growth factor, and (+GF, HK_a) represents proliferation in the presence of both growth factor and HK_a. The significance of differences in 25 relative endothelial cell proliferation cell numbers at the end of the proliferation assays was determined using the Student's two-tailed T-test for paired samples.

B. Results

30 Inhibition of endothelial cell proliferation by HK_a was apparent within 6 hours after its addition to cells, at which time cell spreading appeared to be diminished, and the cells began to display a more rounded morphology. However, the extent to which proliferation was inhibited

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increased progressively with longer exposure of cells to HK_a (Figure 1). Inhibition of endothelial cell proliferation by HK_a occurred in a concentration-dependent manner; modest inhibition (14%) of bFGF (10 ng/ml) - stimulated proliferation occurred at an HK_a concentration of <1.0 nM, while HK_a inhibited proliferation by 50% at a concentration of approximately 8 nM (Figure 2).

Single-chain HK, low molecular weight kininogen and bradykinin were tested in the same manner. HK_a inhibited endothelial cell proliferation to a much greater extent than single-chain HK. Low molecular weight kininogen, which is derived from alternative splicing of the kininogen gene and contains the entire HK light chain, domain 4 and a truncated domain 5 containing only 12 amino acids, had no effect on endothelial cell proliferation (Figure 2). Finally, bradykinin did not inhibit proliferation, and anti-bradykinin antibodies did not inhibit the anti-proliferative effect of HK_a (not shown). The latter results exclude the possibility that contamination of HK_a with trace amounts of bradykinin was responsible for its ability to inhibit endothelial cell proliferation. Single-chain HK modestly inhibited endothelial cell proliferation.

These results demonstrate that HK_a is a potent and rapid inhibitor of endothelial cell proliferation *in vitro*, and that these activities occur within a concentration range similar to that of previously-described anti-angiogenic polypeptides.

HK_a inhibited the proliferation of human endothelial cells in response to a number of mitogens, including bFGF, VEGF, HGF, TGF-β and PDGF, equally well (Figure 3). The mitogenic activity of each of these factors is mediated through interactions with distinct receptors. Thus, without wishing to be bound by any theory, these results imply that the mechanism(s) by which HK_a and the HK_a peptides inhibits endothelial cell proliferation is unlikely to depend upon inhibition of growth factor binding.

HK_a inhibited the proliferation of HUVEC and HDMVEC with similar potency but did not affect the proliferation of human aortic smooth muscle cells (Fig. 4) or HEK 293 cells (not shown), demonstrating that HK_a's anti-proliferative effects were endothelial cell-specific. Furthermore, the ability of HK_a to inhibit endothelial cell proliferation was inversely proportional to the density of the cell culture (Figure 5), suggesting that its

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effects may be at least partially dependent upon the rate of cell proliferation and DNA synthesis.

In other experiments, the ability of HK_a to inhibit the proliferation of endothelial cells cultured on different extracellular matrix (ECM) proteins. HK_a (10nM) potentially inhibited the proliferation of HUVEC cultured on gelatin, laminin and Matrigel, though slightly less potent inhibition, largely overcome by high concentrations of HK_a (50 nM), occurred when cells were cultured on fibronectin or vitronectin. Intermediate effects were observed when cells were cultured on fibrinogen, though cells cultured on collagen types I or IV were resistant to the antiproliferative activity of HK_a.

Example 2

Apoptosis of Endothelial Cells Induced by Two-Chain High Molecular Weight Kininogen

A. Experimental

Induction of endothelial cell apoptosis after exposure of cells to HK_a was determined using three assays.

Cells plated on glass coverslips were cultured in the absence or presence of 30 nM HK_a for periods of 2-24 hours. Cells were then fixed for 1 hour in phosphate-buffered saline (PBS) containing 1% formaldehyde, and stained for 2 hours with a solution containing 1 µg/ml of 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) and 10 µg/ml of sulforhodamine 101 (Molecular Probes, Eugene OR). Stained cells were visualized by UV illumination using a Nikon Microphot FXA microscope (objective 40X, Neofluor). Nuclear condensation, fragmentation and hyperchromaticity were considered to reflect apoptosis.

DNA fragmentation after exposure of cells to HK_a was also directly assessed. Briefly, cells were cultured in the absence or presence of 30 nM HK_a, for varying periods, and DNA isolated following cell lysis in a buffer containing 20 mM Tris-HCl, pH 7.4, 5 mM ethylenediamine tetraacetic acid (EDTA) and 0.4% Triton X-100. After centrifugation to remove nuclei and insoluble material, the supernatant was extracted with an equal volume of phenol:chloroform:1-isopropanol (25:24:1), and DNA precipitated by the addition of 50 µl of 4 M LiCl and 500 µl 2-propanol. Precipitated DNA was dried using a Speed-Vac (Savant, Holbrook, NY),

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resuspended in 20 mM Tris-HCl, pH 7.4, containing 5 mM EDTA and incubated for 30 minutes in the presence of 0.1 mg/ml RNase A. Samples were then analyzed by 0.8% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

5 Apoptosis was also confirmed by using the TUNEL reaction (*In Situ* Cell Death Detection Kit, Boehringer Mannheim, Indianapolis, IN) to label control cells and those exposed to HK_a with fluorescein-conjugated dUTP, per the manufacturer's protocol. Labeled cells were then analyzed by flow cytometry.

10 B. Results

 Staining of cells with DAPI revealed nuclear condensation and fragmentation only in cells that had been exposed to HK_a; these changes were observed in 30-50% of the cells within 6 hours after HK_a addition (Figure 6A). Parallel studies in which cells were incubated with
15 Trypan blue after incubation with HK_a revealed no evidence of dye uptake, demonstrating that HK_a did not induce cell lysis, and that its effects were due to the induction of apoptosis rather than cytotoxicity. Consistent with this observation, analysis of DNA isolated from cells incubated with HK_a revealed striking fragmentation, which was first apparent approximately 6
20 hours after addition of the HK_a (Figure 7A). Consistent with these results, a specific "laddering" pattern of DNA fragmentation, characteristic of apoptosis, was apparent upon electrophoretic analysis of DNA from cells exposed to HK_a (data not shown). Finally, flow cytometric analysis of cells incubated in the absence or presence of HK_a, and labeled with fluorescein
25 dUTP by the TUNEL reaction, and revealed a rightward shift of the major peak only in cell populations exposed to HK_a (not shown). As with DAPI staining and DNA fragmentation, these changes were apparent approximately 6 hours after exposure of cells to HK_a. Taken together, these studies demonstrate that the antiproliferative activity of HK_a reflects
30 its ability to induce endothelial cell apoptosis.

Example 3

**Inhibition of Cytokine-Stimulated Angiogenesis by
Two-Chain High Molecular Weight Kininogen *In Vivo***

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A. Experimental

The effect of HK_a on cytokine-stimulated angiogenesis *in vivo* was determined using a previously-described assay in which the neovascularization of a Matrigel "plug" containing bFGF is assessed (Passaniti *et al.*, *Lab Invest* 67:519-528, 1992). Briefly, athymic Ncr nude mice (7-8 weeks old, females) were injected subcutaneously on the left and right mid-back with 0.25 ml of chilled Matrigel containing 400 ng bFGF and 50 µg heparin, to which either 25 µl of PBS (left mid-back injection) or an equal volume of PBS containing 0.4 mg/ml HK_a (right mid-back injection) had been added. Immediately after injection, the Matrigel solidified and remained as a solid, subcutaneous "plug" through the 4 day duration of the experiment. At this point, mice were sacrificed, and the skin incised along the mid back and peeled back over the flanks to expose the Matrigel plugs. Plugs were then photographed prior to their excision, fixation and processing, as previously described (Passaniti *et al.*, *Lab Invest* 67:519-528, 1992).

The effect of HK_a on cytokine-stimulated angiogenesis *in vivo* was also determined using a rat corneal micropocket angiogenesis assay as previously described (Polverini *et al.*, *Meth. Enzymol.* 198:440-450, 1991; Fournier *et al.*, *Inv. Ophthalm. Vis. Sci.* 21:354, 1981). Pellets were prepared using 12% hydron. Control pellets contained bFGF (50 ng/pellet), while test pellets contained bFGF and HK_a (final concentration 10 µM). A single pellet was implanted in a 2 mm pocket prepared in each cornea, 1 mm from the limbus. The left eye of each animal received the control pellet, while the right eye received the HK_a-containing pellet. Corneal neovascularization was measured after 7 days, at which time a digital image of each eye was obtained using a Nikon NS-1 slit lamp. To determine the total area of neovessels in each eye, digital images were analyzed using a Leica-Qwin (Northvale, NJ) image analysis system (Conrad *et al.*, *Lab. Invest.* 70:434, 1994).

B. Results

As depicted in Figures 8A and 8B, Matrigel plugs containing bFGF induced exuberant vessel ingrowth within 4 days after implantation.

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In contrast, no neovascularization of Matrigel plugs which contained bFGF and HK_a was observed (Figures 8C and 8D). In addition, these plugs remained transparent, as opposed to the opaque appearance acquired by the plugs, suggesting that HK_a blocked the intravasation of migratory cells into the Matrigel. The latter hypothesis was confirmed by histological analysis, which demonstrated markedly fewer cells within the HK_a-containing Matrigel plugs. Furthermore, the cells which had migrated into these plugs appeared rounded and apoptotic, in contrast to the elongated, migratory phenotype of the cells invading the control plugs.

In the corneal micropocket angiogenesis assay, bFGF-containing hydron pellets implanted into control corneas induced a robust angiogenic response. In comparison, the length and density of neovessels were significantly reduced in corneas in which the implanted pellets contained bFGF and HK_a. Computer analysis of digital images revealed that the total vessel area within corneas that received HK_a-containing pellets (293,807 μm^2) was reduced by 82% in comparison to those in which pellets contained bFGF only (53,931 μm^2) ($P < 0.000000005$).

Example 4

Effect of Peptide Analogs of Two-Chain High Molecular Weight Kininogen on Endothelial Cell Proliferation

The following HK_a-derived peptides were synthesized:

Gly-His-Lys-Phe-Lys-Leu-Asp-Asp-Asp-Leu-Glu-His-Gln-Gly-Gly-His (SEQ ID NO:7);

Gly-His-Gly-His-Gly-His-Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn (SEQ ID NO:8); and

His-Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys-Thr (SEQ ID NO:9).

The endothelial cell proliferation assay of Example 1 was performed, utilizing bFGF as the growth factor to stimulate angiogenesis and 50 μM of the above peptides. The same mathematical formula was employed but proliferation in the presence of GF plus peptide substituted for proliferation in the presence of GF plus HK_a. The percent inhibition of

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endothelial cell proliferation attributable to the peptides is given in Table 1. The value for HK_a is also reported. The greater than 100% inhibition level achieved in this assay with HK_a (100% being no endothelial cell proliferation, that is, the level of proliferation in medium containing 2% serum alone, without added growth factor) reflects the fact that HK_a induces endothelial cell apoptosis.

Table 1
Inhibition of Endothelial Cell Proliferation by HK_a and HK_a-Derived Peptides

Inhibitor (50 μ M)	Inhibition of Endothelial Cell Proliferation	IC 50
SEQ ID NO:7	59%	n.d.
SEQ ID NO:8	81%	8 μ M
SEQ ID NO:9	92%	14 μ M
HK _a	135%	10 nM

All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.